Regression of Lung and Colon Cancer Xenografts by Depleting or Inhibiting RLIP76 (Ral-Binding Protein 1)

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Abstract

Ral-binding protein 1 (RALBP1) is a stress-responsive and stress-protective multispecific transporter of glutathione conjugates (GS-E) and xenobiotic toxins. It is frequently overexpressed in malignant cells and plays a prominent antiapoptotic role selectively in cancer cells through its ability to control cellular concentration of proapoptotic oxidized lipid byproducts. In the absence of chemotherapy, depletion or inhibition of RALBP1 causes regression of syngeneic mouse B16 melanoma. Because RALBP1 transports anthracycline and Vinca alkaloid drugs, as well as GS-E, and because it confers resistance to these drugs, we proposed that depletion or inhibition of RALBP1 should cause regression of human solid tumors that overexpress RALBP1 and augment chemotherapy efficacy. Non–small-cell lung cancer (NSCLC) H358 and H520 and colon SW480 cell lines were used. Cytotoxic synergy between anti-RALBP1 immunoglobulin G (IgG), cis-diammine-dichloroplatinum (II) [CDDP], and vinorelbine was examined in cell culture and xenografts of NSCLC cells. Effects of RALBP1 depletion by antisense were examined in xenografts of NSCLC H358, NSCLC H520, and colon SW480 cell lines. RALBP1 depletion by phosphorothioate antisense was confirmed and was associated with rapid, complete, and sustained remissions in established s.c. human lung and colon xenografts. RALBP1 inhibition by anti-RALBP1 IgG was equally as effective as antisense and enhanced CDDP-vinorelbine in lung cancer xenografts. These studies show that RALBP1 is a transporter that serves as a key effector function in cancer cell survival and is a valid target for cancer therapy, and confirm that inhibitory modulation of RALBP1 transport activity at the cell surface is sufficient for antitumor effects. [Cancer Res 2007;67(9):4382–9]

Introduction

The human Ral-binding protein 1 (RALBP1) functions as an antiapoptotic and stress-protective protein that prevents cellular accumulation of toxic metabolites of lipid oxidation products by catalyzing ATP-dependent transport of reduced glutathione (GSH)-electrophile conjugates (GS-E) from cells (1–9). Studies in RALBP1 knockout mice have shown that RALBP1 is essential for the accumulation of toxic metabolites of lipid peroxidation products by catalyzing ATP-dependent transport of reduced glutathione (GSH)-electrophile conjugates (GS-E) from cells (1–9). Studies in RALBP1 knockout mice have shown 2- to 7-fold increased accumulation of lipid hydroperoxides, aldehydes, and alkenals in tissues as a consequence of RALBP1 loss (9). This is accompanied by >80% loss of total transport activity for GS-E as well as anthracycline; this loss translates into an order of magnitude greater sensitivity to xenobiotic toxins including natural product chemotherapy drugs, which are directly transported by RALBP1, as well as alkylating agents and platinum-coordinates that are metabolized to GS-E. Because GS-E formed downstream of lipid peroxidation are common toxins formed from both chemical and radiant stress, RALBP1 loss also confers dramatic sensitivity to apoptosis caused by radiant stressors, which can induce lipid peroxidation, including heat, UV, and ionizing radiation (9–11). As predicted by studies in knockout animals, augmenting RALBP1 in cell culture confers resistance to anthracyclines, Vinca alkaloids, mitomycins, alkylating agents, platinum coordinates, hydrogen peroxide, alkenals, UV light, heat, and X-irradiation (9–11). In animal models, supplementing RALBP1 increased survival time nearly 3-fold after lethal irradiation with 500 GY (9). The central role of RALBP1 function as a stress-protective antiapoptotic protein is also reflected in the number of critical stress signaling proteins found in complexes with RALBP1, including protein kinase C (PKC)-α, cdc2, Ras-R, cdc42, RalA, heat shock protein 70, heat shock protein 90, Hsf-1, POB1, AP2 clathrin adaptor, and epsin (4). That RALBP1 provides an essential effector function regulated by these signaling molecules is supported by recent studies showing that activation or depletion of PKCα has no effect on cell growth in mouse embryonic fibroblasts (MEF) from RALBP1−/− mice (12, 13).

Protection from stress-mediated apoptosis is essential for cancer cells, which often have to survive under very adverse conditions. RALBP1 expression is found to be increased in a number of malignant cells by gene expression array studies.4 Depleting or inhibiting RALBP1 causes apoptosis in cultured cells from a variety of human solid and hematologic malignancies including non–small-cell lung cancer (NSCLC), small-cell lung cancer, melanoma, ovarian cancer, prostate cancer, colon cancer, myeloid leukemia, and lymphoma (14). The relatively greater sensitivity to apoptosis on RALBP1 depletion in malignant as compared with nonmalignant cells in culture is also reflected in the B16 mouse melanoma model, where complete tumor regression occurs after a single i.p. dose of R508 antisense targeted to specifically deplete RALBP1 (14). These results naturally raised the questions of whether these observations would be applicable to human malignancy and whether RALBP1 depletion or inhibition could augment the efficacy of cytotoxic chemotherapy. Present studies address these questions in cell culture and nude mouse xenografts of human lung and colon cancer cell lines. We used the lung and colon cancer xenograft models to test whether RALBP1 depletion by antisense would cause cancer regression and used the lung cancer model in...
cell culture and xenograft to test whether inhibition of RALBP1 by anti-RALBP1 immunoglobulin G (IgG) would enhance the efficacy of cisplatin (a GS-E-forming drug) and vinorelbine (a Vinca alkaloid known to be transported by RALBP1). This chemotherapy combination was felt particularly suited because it is known to be synergistic and is the first combination chemotherapy to be shown to prolong survival in an adjuvant setting.

**Materials and Methods**

**Reagents.** RPMI 1640 and DMEM, PBS, penicillin/streptomycin solution, fetal bovine serum (FBS), trypsin-EDTA, and trypsin blue were purchased from Life Technologies, Inc. Keratinocyte serum-free medium and EGM-2 bullet kit media were purchased from Cambrex BioScience. DMSO, G418 (geneticin), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma. Vinorelbine (Navelbine) and doxorubicin were obtained from GensiaSicor Pharmaceutical and Adria. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and dihydroethidium (DHE) were obtained from Invitrogen (Invitrogen). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and dihydroethidium (DHE) were obtained from Sigma. Vinorelbine (Navelbine) and doxorubicin were obtained from GensiaSicor Pharmaceutical and Adria. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and dihydroethidium (DHE) were obtained from Sigma. Vinorelbine (Navelbine) and doxorubicin were obtained from GensiaSicor Pharmaceutical and Adria.

**Cell lines and cultures.** Human lung cancer cell lines H520 (squamous cell carcinoma), H358 (Bronchio alveolar), H2347 (adenocarcinoma), and colon cancer cell line SW480 were purchased from American Type Culture Collection. Human lung bronchioepithelial cells (HLBEC), human umbilical vascular endothelial cells (HUVEC), and human aortic vascular smooth muscle cells (HAVMSC) were kindly provided by Drs. John D. Minna and Fiemu Nwariaku (University of Texas Southwestern Medical Center, Dallas, TX), respectively. All cells were cultured at 37°C in a humidified atmosphere of 5% CO2 in the appropriate medium: RPMI 1640 (H358, H520, H2347, and SW480), DMEM (HAVMSC), keratinocyte serum-free medium (HLBEC), and EGM-2 bullet kit (HUVEC) medium supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin solution, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate.

**Transfection of RALBP1 in NSCLC cells.** H358 (NSCLC) were transfected with the eukaryotic expression vector alone (pcDNA3.1) or with pcDNA3.1-RALBP1 using Effectene Transfection Reagent kit (Qiagen). Expression of RALBP1 mRNA was evaluated by reverse transcription–PCR analysis. The DNA was prepared with RNeasy kit (Qiagen). RNA was quantified and purity was determined by measuring absorbance at 260 and 280 nm. RALBP1 gene-specific primers (334–353 bp (upstream primer) and 1,209–1,228 bp (downstream primer)) were used for RT-PCR. Ready-to-go RT-PCR beads were used according to the manufacturer’s instructions (Amersham Biosciences). Levels of RALBP1 protein in control and transfected clones were measured by immunoblotting using anti-RALBP1 IgG. Aliquots of crude detergent membrane fraction of cells containing 200 μg protein were applied to SDS-PAGE and Western blot analyses were done as previously described (8).

**Anti-RALBP1 antibodies.** We have raised and purified polyclonal rabbit anti-human RALBP1 IgG (also referred to previously as anti-FLIPL76 IgG) using procedures previously described (2) and aliquots were stored at −86°C. All reagents for the preparation of antibodies and storage were filtered through 0.22-μm filters and handled under laminar flow hoods in a sterile manner. Aerobic, anaerobic, and fungal cultures of random aliquots were done at 2-month intervals. The integrity and purity of the antibodies were consistently checked by SDS-PAGE and Western blot analysis against anti-IgG antibodies during these studies. Protein A affinity-purified immunoglobulin fraction obtained from the preimmune serum was used as control. Anti-RALBP1 IgG used in these experiments was previously shown by Ouchterlony double immunodiffusion assay to be non–cross-reactive with any other proteins including G-protein or MRP1 (4, 5).

**Drug sensitivity assay.** Cell density measurements were done with a hemocytometer to count reproductive cells resistant to staining with trypan blue. Approximately 2 × 10^5 cells were plated into each well of a 96-well flat-bottomed microtiter plate 24 h before addition of medium containing varying concentrations of either preimmune serum or anti-RALBP1 IgG (0–10 μg/ml final concentration). After 24-h incubation, 40-μL aliquots of drug (vinorelbine or CDDP and both; concentrations ranging from 1 to 10,000 nm) were then added to eight replicate wells to assess the IC50 of drug, which is defined as the concentration at which formazan was reduced by 50%. After 96-h incubation, 20 μL of 5 mg/ml MTT were introduced to each well and incubated for 2 h of exposure. The plates were centrifuged and medium was decanted. Cells were subsequently dissolved in 100-μl DMSO with gentle shaking for 2 h at room temperature, followed by measurement of absorbance at 570 nm (15).

**Chou-Talalay median effect analysis of synergy.** The Chou-Talalay method of determination of synergy (16) is based on measurement of IC50 values for each drug alone and in combinations. The Calcsyn software package, which uses an algorithm that takes into account the cytotoxicity as well as dose-effect curves for each chemotherapy drug and the antibodies, was used. Analysis of synergy was done by determining the combination index from the isobologram equation for two drugs or three drugs. Combination index < 1, combination index = 1, and combination index >1 correspond to synergy, additivity, and antagonism, respectively.

**Efficacy studies of H3-vinorelbine in the presence of CDDP and anti-RALBP1 IgG.** The NSCLC (H358) cells were harvested and washed with PBS. Aliquots containing 5 × 10^5 cells were inoculated into fresh medium. Anti-RALBP1 IgG (40 μg/ml final concentration) alone or in combination with CDDP (1 μmol/L final concentration) were added to the cells in microcentrifuge tubes and incubated at 37°C for 60 min in a CO2 incubator. The cells were pelleted and resuspended in 90-μL medium. 1H-Vinorelbine (100 nmol/L; specific activity, 4,080 cpm/pmol) was then added to the medium. The cells were then incubated for 60 min at 37°C. Cells were centrifuged at 300 × g for 5 min, after which the supernatant was removed completely and the cell pellet washed with PBS twice. The pellet was immediately resuspended in 1 mL of PBS. Fifty-microLiter aliquots of clear supernatant were removed every 60 s for radioactivity counting for 15 min. The back-added curves of cellular residual vinorelbine versus time were constructed as previously described (8, 17).

**Immunohistochemical analysis.** Buffered formalin-fixed, paraffin-embedded tissue sections (5 μm) were deparaffinized and rehydrated by passage through xylene and graded ethanol solutions. Slides were then treated with 3% hydrogen peroxide with 0.03% sodium azide in PBS for 10 min, followed by microwave antigen retrieval at 100°C for 10 min in DAKO Target Retrieval Solution (DAKO Corp.) in an H2800 Microwave Processor (Energy Beam Sciences, Inc.). Slides were incubated in 0.05% casein (Sigma)/0.05% Tween 20/PBS for 30 min to block nonspecific protein binding. Rabbit anti-human RALBP1 IgG was applied to sections at a 1:200 dilution for 60 min. Universal Negative Control for rabbit primary antibodies (DAKO) was used as negative control. Envision+ System labeled polymer-horseradish peroxidase antirabbit was used for detection and color was developed with 3,3'-diaminobenzidine (Dako). Slides were counterstained with Mayer’s modified hematoxylin (Poly Scientific) before mounting and viewed under an Olympus BX51 microscope; images were recorded by a DP70 digital camera (Diagnostic Instruments, Inc.).

**Animal model.** Hsd: athymic nude nu/nu mice were obtained from Harlan. All animal experiments were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Fifty-one 14-week-old mice were divided into 17 groups of 3 animals (treated with PBS, preimmune IgG, scrambled antisense DNA, anti-RALBP1 IgG, R508, CDDP, vinorelbine, and with various combinations). Thirty-three animals were injected with 2 × 10^6 H358 NSCLC, nine animals were injected with 2 × 10^6 H520 NSCLC, and nine animals were injected s.c. with 2 × 10^6 SW480 colon cancer cell suspensions in 100 μL of PBS. Animals were examined daily for signs of tumor growth. Treatment was administered when the tumor surface area exceeded 45 mm2 (days 22 and 25 for lung and colon cells, respectively). Treatment consisted of 200 μg anti-RALBP1 IgG, R508, 6 μg vinorelbine, 30 μg CDDP, 6 μg vinorelbine + 30 μg CDDP, 200 μg...
anti-RALBP1 IgG + 30 μg CDDP, 200 μg anti-RALBP1 IgG + 6 μg vinorelbine, and 200 μg anti-RALBP1 IgG + 30 μg CDDP + 6 μg vinorelbine, in 100 μL PBS, i.p. Control groups were treated with 200 μg/100 μL preimmune IgG (preimmune serum), scrambled antisense, or diluent (PBS) alone. Tumors were measured in two dimensions using calipers.

**Immunohistochemistry and TUNEL assay on excised xenografts.** Nude mice bearing lung cancer tumors (size ~50 mm²) were injected i.p. with a single dose of 200 μg of either scrambled or R508 and sacrificed after 48 h. S.c. tumors were excised, rinsed with PBS containing 100 units/mL of penicillin and 0.1 mg/mL of streptomycin, minced in 2 μL of trypsin/EDTA, and incubated at 37°C for 30 min. After further disruption by repeated aspiration in a 1-mL syringe, cells were digested in RPMI 1640 containing 10% FBS. The cells were allowed to attach to coverslips by overnight incubation in RPMI 1640 containing 10% (v/v) heat-inactivated FBS, 1% (v/v) penicillin/streptomycin solution, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate at 37°C in a CO₂ incubator. Effect of depletion of RALBP1 by R-508 on immunohistochemistry using anti-RALBP1 IgG and apoptosis by TUNEL assay were done according to the method previously described (18, 19).

**Results**

**Antineoplastic effects of RALBP1 depletion by phosphorothioate antisense DNA.** We have previously shown that RALBP1 depletion by the phosphorothioate antisense R508 (directed against nucleotides 508–528 of RALBP1) induces apoptosis in cultured human malignant cells, including melanoma, lung cancer, prostate cancer, and ovarian cancer, and causes complete regression of established s.c. murine B16 melanoma (14). To determine whether these findings are applicable to human cancer cell lines, we compared the cytotoxicity of R508 between immortalized nonmalignant cells, including HLBEC, HUVEC, and HAVSMC cells, versus malignant cell lines, including human lung, colon, ovary, and melanoma and mouse melanoma (Table 1). The malignant cells were significantly more susceptible than nonmalignant cells to the cytotoxicity of R508. Encouraged by these results, we subsequently tested the ability of R508 to cause regression of xenografts of NSCLC H358, NSCLC H520, and colon cancer SW480 cell lines. The ability of R508 to deplete RALBP1 in mouse tissues was first examined. Non–tumor-bearing animals injected with a single 200-μg dose of R508 were sacrificed at 1, 2, 4, and 14 days later and crude homogenate fractions of liver, kidney, lung, and brain were assayed for RALBP1 protein by ELISA (18) and for total protein by Bradford assay. RLIP76 represented a remarkably constant 0.4 ± 0.05% of total extractable protein in all four organs (Fig. 1A). This amount is comparable to the ~0.6% previously reported in lung and other cancer cells (14, 18). Within 24 h of R508 treatment, RALBP1 was depleted to <0.01% of the total extractable protein and a slow partial recovery was seen within a 14-day period of these experiments. Immuno histochemical studies for RALBP1 expression further confirmed ELISA findings. Representative findings from immunohistochemistry in kidney tissue are presented (Fig. 1B). These results indicated that the R508 regimen used during the present studies caused RALBP1 depletion in tissues.

Tumor-bearing animals with established s.c. implanted tumors >45 mm² were treated with 200 μg of R508 administered on days 1 and 15 by i.p. injection. R508-treated animals had rapid and dramatic reductions in tumors until disappearance before day 20; the remarkable contrast in the outcome of tumors in animals treated with R508 versus scrambled phosphorothioate was clearly evident for two lung cancer cell lines and one colon cancer cell line (Fig. 1C–E). The R508-treated mice with lung or colon cancer are still alive at >10 months without evidence of recurrence (see Supplementary data). Weight gain in R508 animals was comparable to non–tumor-bearing controls, and no overt toxicity was evident, perhaps the clearest demonstration of sufficiently cancer-specific apoptotic activity of R508 in the present model system. To examine whether this depletion also occurred in the tumor and to determine whether the resultant disappearance of tumor was due to triggering of apoptosis, we excised tumors from flanks of animals 48 h after i.p. dosing of R508 and examined the cells for the presence of RALBP1 protein by immunohistochemistry and for the occurrence of apoptosis by TUNEL stain. As shown in Fig. 1F, RALBP1 is immunohistochemically detectable in the tumor from animals treated with the scrambled antisense, in which there is also little or no apoptosis identified by TUNEL assay. This is in stark contrast with R508, which nearly completely depleted RALBP1 and caused extensive apoptosis (Fig. 1F).

**Antineoplastic effects of RALBP1 inhibition, CDDP, and vinorelbine.** The above studies establish that RALBP1 depletion causes regression of lung and colon cancer xenografts but do not distinguish between two possible models of RALBP1 function: that the transport activity of RALBP1 is the key element necessary for its protective effects or that, as proposed by others, it functions through signaling interactions with antiapoptotic signaling proteins distinct from its transport activity. Because R508 depletes virtually all cytosolic and membrane-associated RALBP1, the antineoplastic effect would be the same with either of the two models. We reasoned that an experimental approach aimed at specifically inhibiting transport activity of RALBP1 without affecting total cellular RALBP1 would be necessary to distinguish between the two models. We have previously shown that a cell-surface epitope of RALBP1 (amino acids 171–185) is recognized by anti-RALBP1 IgG as well as by polyclonal antibodies generated.
against the specified peptide, and that this recognition is abrogated on sole expression of mutant RALBP1 lacking this peptide (19). Coating live cells with either antibody causes rapid increase in cellular content of transported substrates including doxorubicin and 4-hydroxynonenal-glutathione conjugate (4HNE-SG; refs. 7, 10, 11), whereas total RALBP1 protein content remains unchanged. These findings predict that anti-RALBP1 IgG should function with at least equal antitumor efficacy as the antisense if the anti-apoptotic activity of RALBP1 is dependent on its transport activity.

The specificity of the present anti-RALBP1 IgG was clearly apparent from studies showing that the antibody recognizes only two peptides, 95 and 38 kDa, in crude homogenate of mouse heart tissue from wild-type mouse, and no peptides were recognized in the similarly prepared homogenate from the RALBP1−/− animal, which by definition lacks RALBP1. The striking lack of any recognized band in the RALBP1−/− could not be overcome by increasing protein loaded per well from 150 to 400 μg (Fig. 2A). The 95-kDa band represents intact RALBP1, confirmed by NH₂-terminal sequence analyses, and the 38-kDa band is an internal fragment beginning at residue Ser²²⁵. These results showed essentially absolute specificity of these antibodies in the mouse model. To test this further in the human cells, we similarly applied crude protein samples (200 μg/well) from three lung cancer, one colon cancer, and two immortalized nonmalignant cell lines (Fig. 2B). As previously seen (5), only the 95- and 67-kDa bands are seen in these cells; both bands were transblotted onto nitrocellulose membrane and shown by amino acid NH₂-terminal sequencing to be the NH₂-terminal and the internal peptide beginning at residue Met²²⁵ characteristic for cultured cell lines (5), whereas the 95- and 38-kDa bands are always observed in RALBP1 purified from tissues (2, 9). These differences may be related to both differential posttranslational modifications, as well as to known splice variants of RALBP1 (5). Lack of any significant extraneous bands in these studies attests to the relative specificity of these antibodies in human

Figure 1. Antineoplastic effect of RALBP1 depletion by R508 phosphorothioate antisense. A, time course of RALBP1 depletion by ELISA assay. Wild-type animals were sacrificed at 24 h, 48 h, 96 h, and 2 wks after one dose of R508 (200 μg in 100 μL of PBS). I.p. Control groups were treated with 200 μg of scrambled antisense phosphorothioate in 100 μL of PBS (three animals per group). Liver, lung, kidney, and brain tissues were removed from animals at each time point. RALBP1 protein content was measured in homogenates prepared from each tissue by an ELISA assay using anti-RALBP1 IgG as previously described by us for cultured cells (18). B, time course of RALBP1 depletion in renal tissue by immunohistochemistry. Studies were done on kidney tissue at each time point with anti-RALBP1 IgG as a primary antibody using Mayer’s modified hematoxylin counterstaining as previously described (9). C to E, antineoplastic effect of RALBP1 depletion in NSCLC H358, H520, and colon cancer SW480 xenografts. An aliquot of 2 × 10⁶ H358, H520, or SW480 cells in 100-μL PBS was injected s.c. into one flank of each nu/nu nude mouse (three animals per group). When tumors reached a cross-sectional area of ~45 mm², animals were randomized into treatment groups as indicated in the figure. Tumors were measured in two dimensions using calipers. F, effect of depletion of RALBP1 by R-508 on immunohistochemistry and apoptosis by TUNEL assay. Nude mice bearing lung cancer tumors (size ~ 50 mm³) treated with 200 μg of either scrambled or RALBP1 R508 were dissected after 48 h and primary cultures from both scrambled- and RALBP1 R-508–treated tumors were prepared. For immunohistochemical localization studies, anti-RALBP1 IgG was used as primary antibody and FITC-conjugated goat anti-rabbit IgG was used as secondary antibody. Induction of apoptosis is shown by TUNEL assay in which green fluorescence represents apoptotic cells.

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systems as well. These results also confirm our previous finding of the significantly lower content of RALBP1 in the immortalized as compared with malignant cell lines (14).

The CDDP-vinorelbine combination is highly active in lung cancer and has been shown by others to be synergistic. A prediction from our recent studies, showing that RALBP1 can mediate efflux of vinorelbine, was that the efficacy of the CDDP-vinorelbine combination could be enhanced by depleting or inhibiting RALBP1. This postulate was tested in cell culture by comparing vinorelbine efflux from wild-type or RALBP1-overexpressing H358 NSCLC cells in the presence of anti-RALBP1 and CDDP (Fig. 2C). Efflux of $^3$I-vinorelbine from cells loaded with the drug was monitored using a modified flow-cell method to quantify time-dependent loss of drug from cells (17). RALBP1-overexpressing cells showed faster vinorelbine efflux than the wild-type or empty vector–transfected cells. Coating cells with anti-RALBP1 IgG markedly reduced vinorelbine efflux but did not abrogate it. Addition of CDDP caused further significant decrease in vinorelbine efflux (Fig. 2C). These results indicated that residual vinorelbine efflux after blocking RALBP1 is likely mediated by ABC transporters, and that addition of CDDP (with resultant formation of GS-CDDP conjugates) likely blocked those ABC transporters known to transport both vinorelbine and GSH conjugates of CDDP, such as MRP1 and BCRP (20, 21). These studies confirm that efflux of vinorelbine is augmented by increasing RALBP1; that it is inhibited partially by anti-RALBP1 IgG; and that this inhibition is more effective in the presence of cisplatin, a GS-E forming agent.

Figure 2. Comparison of antineoplastic effects of CDDP, vinorelbine, and anti-RALBP1 IgG in NSCLC. A, effect of RALBP1 genotype on heart tissue RALBP1 protein is shown by Western blot analysis, with application of 150 μg (lanes 1 and 2, wild-type and knockout homozygous, respectively) and 400 μg (lanes 3 and 4, wild-type and knockout homozygous, respectively) of crude membrane fraction to SDS-PAGE (top) and using anti-RALBP1 IgG as primary antibody and β-actin as internal control (bottom). B, antibody specificity in human cells was examined by Western blotting of crude detergent extracts of the membrane fractions of human lung (H588, H585, and H2347), colon (SW480), HUVEC, and HLBEC cell lines against anti-RALBP1 IgG. The lanes contained 200-μg proteins and Western blot was developed with horseradish peroxidase–conjugated goat anti-rabbit IgG as secondary antibody and 4-chloro-1-napthol as chromogenic substrate. β-Actin was used as internal control. W.B., Western blot. C, effects of RALBP1 overexpression, anti-RALBP1 IgG, and CDDP on vinorelbine (VRL) efflux. H358 wild-type (●), vector alone–transfected (▲), and RALBP1–transfected (◆) cells were loaded with 100 nmol/L radiolabeled $^3$I-vinorelbine and incubated for 60 min; extracellular drug was washed off with PBS, followed by rapid dilution in drug-free buffer. The cells were divided into three groups: untreated (yellow), treated with anti-RALBP1 IgG (green), and treated with anti-RALBP1 IgG and CDDP (brown). Aliquots of buffer were removed every minute and total residual radioactivity in the dilution buffer was measured at the end of the study. Efflux studies were done with three replications in two independent experiments. Points, mean (n = 6). Back-addition curves of cellular residual vinorelbine versus time were constructed as previously described (8, 17). D, in vitro cytotoxic interactions of CDDP, vinorelbine, and anti-RALBP1 IgG. Cytotoxic effects were calculated for absorbance values obtained from MTT cytotoxicity assays. Cells were treated alone or in combinations with vinorelbine (1 nmol/L), CDDP (1 μmol/L), and anti-RALBP1 IgG (20 μg/mL) for 24 h before MTT assay. Eight replicates were done in three separate experiments. E, in vivo antineoplastic interactions of CDDP, vinorelbine, and anti-RALBP1 IgG in xenografts of human NSCLC H358. An aliquot of 2 × 10^6 H358 cells in 100-μL PBS was injected s.c. into one flank of each nu/nu nude mouse (three animals per group). When tumors reached a cross-sectional area of ~ 45 mm^2, animals were randomized into treatment groups as indicated: PBS; preimmune serum; anti-RALBP1 IgG; vinorelbine; CDDP; vinorelbine + CDDP; anti-RALBP1 IgG + CDDP; anti-RALBP1 IgG + vinorelbine; and anti-RALBP1 IgG + vinorelbine + CDDP. Tumors were measured in two dimensions using calipers.
The cytotoxic effects of these drug-combinations were tested in three NSCLC cell lines: H520 (squamous cell carcinoma), H358 (bronchioalveolar), and H2347 (adenocarcinoma). Results of one fixed dose combination are shown (Fig. 2D). The CDDP-vinorelbine effect was supra-additive, as was the CDDP-vinorelbine-anti-RALBP1 IgG effect; vinorelbine-anti-RALBP1 IgG and CDDP-anti-RALBP1 IgG were additive or subadditive. Chou-Talalay analyses of synergy using multiple combinations of dose effects for the three agents confirmed synergy for combinations CDDP-vinorelbine and CDDP-vinorelbine-RALBP1 IgG and indicated additive effects for the vinorelbine-anti-RALBP1 IgG and CDDP-anti-RALBP1 IgG combinations. The predictions from this in vitro model were tested in a nude mouse xenograft model using NSCLC H358. Animals were treated with i.p. injection of 100-μL PBS containing CDDP (1 mg/kg body weight); vinorelbine (0.2 mg/kg of body weight), anti-RALBP1 IgG (7 mg/kg of body weight), or combinations when implanted tumor size reached 45 mm², and repeated 1 and 3 weeks later (Fig. 2E and Supplementary figures). The CDDP-vinorelbine combination was more effective than either drug alone with respect to rate of regression. Remarkably, the animals treated with anti-RALBP1 IgG responded much more quickly than animals treated with CDDP, vinorelbine, or CDDP-vinorelbine. Addition of either CDDP or vinorelbine individually to anti-RALBP1 IgG did not result in any increase in the efficacy of the antibody alone. However, treatment with all three agents caused the most rapid remission. Whereas the chemotherapy-treated animals all died of recurrent disease between 72 and 120 days, 8 of 12 animals receiving any anti-RALBP1 IgG are surviving at >330 days.

These striking results not only confirm the validity of RALBP1 as a target but also strongly support our model for RALBP1 in which it functions to protect from stress through its transport activity. These results also show for the first time that anti-RALBP1 IgG alone exerts antiproliferative activity in xenograft models of human NSCLC at least equivalent to that of a fixed combination of CDDP and vinorelbine. The lack of additional effect of vinorelbine and CDDP on the effect of anti-RALBP1 IgG alone further suggests that inhibition of efflux of physiologic GS-E (such as 4HNE-SG) may be an important mechanism for the apoptotic effects of vinorelbine or CDDP; i.e., these drugs exert at least some of their apoptotic effect by competitive inhibition of physiologic lipid transport by RALBP1. Accumulation of such ligand, GS-HNE, is known to directly inhibit GSH-linked metabolism of lipid hydroperoxides and their potent proapoptotic alkenal derivatives, particularly 4HNE. In context of such a model, it would not be surprising to see no additional effect of either of these drugs alone when RALBP1 is inhibited. The increased anti-RALBP1 IgG effect in the presence of both drugs may be related to the presence of distinct but related overlapping sites on RALBP1 for binding GS-E versus natural product toxins; thus, perhaps the presence of both drugs is more effective at inhibiting efflux of physiologic ligands (GS-E formed from lipid hydroperoxides).

Discussion

During present studies, we show that i.p. administration of RALBP1 antisense or anti-RALBP1 IgG to nude mice leads to complete and sustained regression of lung (NSCLC) and colon cancer xenograft tumors. These findings indicate that RALBP1 is a key survival protein for NSCLC and colon cancer cells, and that its depletion/inhibition results in regression of human NSCLC and colon cancer xenografts without any apparent toxicity to animals. Because both anti-RALBP1 antibodies and R508 antisense DNA produce similar effects, our findings support the assertion that the antiapoptotic and stress-protective effects of RALBP1 are primarily related to its transport activity. RALBP1 is present in cytosol, and for its transport function, it can also anchor to membrane (19). Functions attributed to the intracellular RALBP1 (e.g., its signaling role as a Ral or Ras-R GTPase-activating protein; refs. 22–26) are least likely to be affected by anti-RALBP1 antibodies that cannot enter the cells and specifically interact with epitopes on membrane-anchored RALBP1 to inhibit its transport function. In case the lack of presence of RALBP1 within cells also contributes to the observed suppression of tumors due to the cessation of some or all of its known cellular functions (e.g., involvement in Ras-R signaling, endocytosis and mitosis, spindle motor functions, and the regulation of the expression of heat shock proteins), one would expect a more tumor-suppressive activity associated with antisense as compared with anti-RALBP1 IgG. Our findings show that RALBP1 is a unique anticancer target that is a versatile and efficient multidrug transporter and an antiapoptosis protein essential for the survival of cancer cells. Through its ability to efficiently transport the GSH conjugates of endogenous proapoptotic molecules (e.g., 4HNE), as well as chemotherapeutic drugs, it provides survival advantage to cancer cells.

Multiple signaling mechanisms seem to operate in parallel under conditions of oxidative stress to induce apoptosis and to defend against it (10, 11, 27–29). Free radicals activate cellular stress-sensitive pathways including e-Jun NH₂-terminal kinase, p38, IκB kinase, nuclear factor κB (NF-κB), and extracellular receptor kinases (10, 11, 30–32), leading to the production of superoxide anion radicals by NADPH oxidase (33–35). The availability of superoxide anion accelerates lipid peroxidation, resulting in amplification of parallel chemical signaling cascade consisting of oxidized lipids and their obligatory degradation products. 4HNE is the most abundant reactive alkenal formed from lipid peroxidation and is capable of functioning as a toxic endogenous alkyllating agent (29, 36). Lowering cellular 4HNE levels increases proliferation, whereas increasing its concentration triggers differentiation, apoptosis, and necrosis (37). 4HNE is metabolized primarily to the glutathione conjugate GS-HNE by glutathione S-transferase (GST), particularly the α-class isoenzyme A4-4. hGSTA4 directly regulates cellular 4HNE level (38, 39) such that its overexpression in HLEB3 cells depletes 4HNE and causes a dramatic phenotypic transformation accompanied by loss of Fas; down-regulation of p53, p21, p16, fibronectin 1, laminin γ1, connexin 43, integrin α₅β₁ transforming growth factor (TGF)-α, and c-jun; and up-regulation of protein kinase C(α)-2, PKC(β)-2, c-myc, cyclin-dependent kinase 2, and TGF-β1 (40). These results support the direct involvement of 4HNE in carcinogenic transformation. The next step in 4HNE metabolism is the aldose-reductase–catalyzed reduction of GS-HNE to GS-glutathionyl-1, 4-dihydroxynonenol (DHN). Aldose-reductase depletion causes disappearance of GS-DHN, accompanied by inhibition of proliferation of the SW-480 colon cancer cells in culture and arrest of colon cancer xenograft growth in vivo. The mechanism for this effect has been identified as inhibition of key proliferation signals including Cox-2, PKC(γ)-2, phospholipase C, phosphatidylinositol 3-kinase, prostaglandin E2, NF-κB, AP1, interleukin (IL)-1, IL-6, IL-10, IFN-γ, cyclic AMP, MCP (chemokine), and inducible nitric oxide synthase (41, 42).

Aldose-reductase regulates the GS-HNE/GS-DHN ratio, but the absolute cellular concentrations of these and other GS-E are determined by the ATP-dependent transporters, such as RALBP1.
GS-E transporters include the ABC-proteins MRP1 and BCRP, but RALBP1 (RLIP76) is the dominant GS-E transporter (9, 43). This is evident from loss of ~80% transport activity for GS-E in RALBP1 homozygous mouse tissues and from sensitivity of these mice to GS-E–generating stresses including X-irradiation and oxidant chemicals (9). Acute depletion of RALBP1 by siRNA or antisense or inhibition of transport by cell-surface epitope–directed antibodies causes apoptosis in cancer cells selectively, sparing nonmalignant cells (14). Unlike aldose-reductase inhibition, which causes gradual partial response, RALBP1 depletion or inhibition in animal models causes rapid, complete, and sustained regression of malignancy shown here in human xenografts and previously in syngeneic mouse melanoma (14). The independence of the apoptotic effect of RALBP1 from either p53 or ras alterations; greater apoptotic sensitivity of RALBP1–/– MEFs to stress despite constitutively active Akt, c-jun NH2-terminal kinase, AP1, and NF-κB; and complete loss of PKCo-mediated signaling of proliferation and drug-resistance (12, 13) further show the central importance of RALBP1 as an antiapoptotic effector. The efficacy of anti-RALBP1 IgG was superior to either CDDP or vinorelbine alone, highlighting the importance of RALBP1 as an anticancer target and suggesting that apoptosis triggered by CDDP or vinorelbine could be mediated by competitive inhibition of transport of physiologic substrates such as oxidative metabolism–derived GS-HNE, as specifically shown previously in transport studies using artificial and cell membrane–derived liposomes (1). The greater activity of the three-drug combination suggests that, when given together, the two chemotherapy drugs may be more effective at inhibiting not only RALBP1 but also other ABC family transporters. That anti-RALBP1 IgG did not completely abrogate vinorelbine efflux and an increased inhibition was seen when CDDP was present as well support the results of previous studies in which a significant role of ABC transporters such as ABCG2 or ABCB1 has been shown in the transport of vinorelbine as well as GS-CDDP conjugate (20, 21, 43).

In conclusion, present studies show that inhibition or depletion of RALBP1 exerts antineoplastic effects in colon and lung cancer, and indicate that GS-E and xenobiotic transporters occupy a very important position in the hierarchy of stress defense mechanisms necessary for cancer cell survival.

Acknowledgments

References

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Regression of Lung and Colon Cancer Xenografts by Depleting or Inhibiting RLIP76 (Ral-Binding Protein 1)

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